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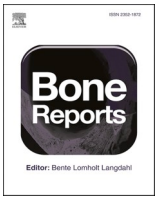

Role of cartilage and bone matrix regulation in early equine osteochondrosis☆,☆☆

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ABSTRACT

The objective of this study is to better understand the pathogenesis of early equine osteochondrosis (OC) by identifying differences in gene and protein expression of extracellular matrix components and regulators in normal and diseased cartilage and bone, focusing on the osteochondral junction and cells surrounding the cartilage canals. We expected to find an upregulation of matrix metalloproteinases and a decrease in extracellular matrix constituent expression along the osteochondral junction and cells surrounding the cartilage canals in OC samples. Paraffin-embedded osteochondral samples (6 OC-affected, 8 normal controls) and cDNA from chondrocytes captured with laser capture microdissection from frozen sections (4 OC-affected, 5 normal controls) were used in this study. Quantitative real-time polymerase chain reaction was performed on 16 target genes. Immunohistochemistry was performed on osteochondral samples for Sox-9, lubricin, osteocalcin, and collagen type IIB. In OC-affected samples, there was significantly ($P \leq 0.05$) decreased gene expression of collagen type IIB, aggrecan, and SOX-9 in chondrocytes surrounding the cartilage canals and decreased gene expression of PRG4 (Lubricin) and collagen type IIB in chondrocytes along the osteochondral junction. We found significantly lower collagen type IIB total matrix percentages in the middle and deep cartilage layers, lower lubricin total cellular percentage in the superficial layer, and higher Sox-9 total cellular percentage in bone of OC samples. No significant differences were found in matrix degradation molecules or HSCORE protein expression at any locations between normal and OC-affected samples in our study.

1. Introduction

Osteochondrosis (OC) is a well-recognized disorder across multiple species resulting from disruption of endochondral ossification during periods of growth (Semevolos, 2017). The majority of research in this area focuses on the complex etiology of clinical disease which involves nutrition, biomechanics, and genetics (Semevolos, 2017). Understanding the pathogenesis of early osteochondrosis prior to clinical manifestation of disease is vital (Laverty and Girard, 2013). During this critical period of growth, several biomechanically weakened areas have been identified within the epiphyseal articular cartilage complex, including the effects of shearing forces along the osteochondral junction and the failure of cartilage canals (Pool, 1993; Olstad et al., 2011). Studies have explored the early pathogenesis of osteochondrosis, with particular focus on failure of vascular anastomoses at the cartilage canals, alterations in biomechanical forces around the cartilage canals, and regulation of extracellular matrix pathways (Laverty and Girard, 2013; Olstad et al., 2013; Pool, 1993).

An ex vivo study evaluating 7-to 12-month old foals finds increased type II collagen cleavage by collagenases in the extracellular matrix of OC-affected explants (Laverty et al., 2002). Another study reports a reduction in total type II collagen content and number of collagen crosslinks in 5- and 11-month old foals having osteochondrosis (van de Lest et al., 2004). Additionally, gene expression of matrix metalloproteinase-13 and -3 is increased in whole cartilage samples of OC-affected foals 1- to 6-months of age (Riddick et al., 2012). Based on these studies, extracellular matrix signaling pathways are expected to be altered in early OC, as they control matrix production and degradation associated with endochondral ossification.

Therefore, the objective of the current study is to extend this

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knowledge by evaluating gene and protein expression of extracellular matrix components and regulators in both normal and OC-affected adolescent osteochondral specimens, focusing on chondrocytes surrounding the cartilage canals and osteochondral junction. The hypothesis of this study is that, when compared to normal samples, OC-affected samples are expected to have an upregulation of matrix metalloproteinases and decrease in extracellular matrix constituent gene and protein expression within cells surrounding the cartilage canals and along the osteochondral junction.

2. Materials and methods

2.1. Sample collection

Paraffin-embedded equine osteochondral samples (6 OC-affected, 8 normal controls) and cDNA from equine chondrocytes captured with laser capture microdissection (4 OC-affected, 5 normal controls) were used in this project, previously obtained from the lateral trochlear ridges of femoropatellar joints in foals ranging from 1 to 6 months of age (see Table 1) (Riddick et al., 2012; Semevolos et al., 2018). A previous study (Riddick et al., 2012) received approval by the Institutional Animal Care and Use Committee (ACUP #3637) for humane euthanasia and collection of samples. Foals were euthanized for reasons unrelated to lameness or joint sepsis using sodium pentobarbital (105 to 187 mg/kg IV).

At the time of harvest, osteochondral samples (n = 2 per trochlear ridge, 3-4 mm thick) were sharply dissected from mid-lateral trochlear ridges of both distal femurs. Cartilage was sharply cut with a scalpel down to bone and then a sharp thin osteotome was used to section the bone underneath. Osteochondral samples were either frozen in OCT medium (Tissue Tek OCT compound, VWR International, Radnor, PA, USA) and stored at -80 °C for laser capture microdissection, or fixed in 4 % paraformaldehyde for 48 h and transferred to 10 % EDTA solution for decalcification (2–4 weeks). Decalcified samples were embedded in paraffin and sectioned for immunohistochemistry and H&E staining (Histopathology Shared Resource Laboratory, Oregon Health & Science University, Portland, OR, USA).

2.2. Sample evaluation and classification

All osteochondral samples were evaluated grossly at the time of harvest and histologically following H&E staining in order to classify them as normal or OC-affected, as previously described (Riddick et al., 2012). Normal cartilage was defined as having no gross or histologic abnormalities. OC-affected was defined as samples having altered endochondral ossification (locally thickened cartilage only, loss of normal columnar arrangement of chondrocytes, chondrones) or separation (fissures, necrosis) along the osteochondral junction without concurrent superficial cartilage lesions (van Weeren and Barneveld, 1999). Briefly, 6 foals were determined to have OC and 8 were classified as normal. In OC samples, 5 foals had separation along the osteochondral junction and 3 foals had locally thickened cartilage (2 with concurrent osteochondral separation), all without concurrent superficial lesions.

2.3. Laser-capture microdissection (LCM)

Frozen osteochondral samples (4 OC, 5 normal) were sectioned using a cryomicrotome, mounted on slides using a tape transfer system (CryoJane, Instrumedics, Leica Biosystems, Inc., Buffalo Grove, IL, USA) and stored at -80 °C. Immediately prior to LCM, each slide was dehydrated in graded alcohol and xylene. LCM was performed using PIXCELL II Laser Capture Microdissection System (Arcturus Bioscience, Mountainview, CA, USA) and CapSure Macro LCM caps (Applied Biosystems, Foster City, CA, USA). Chondrocytes were captured immediately surrounding the cartilage canals, representing small rounded chondrocytes, and osteochondral junction, representing hypertrophic chondrocytes of each animal. Up to 8 caps from sequential sections were combined for each site (approximately 400–800 cells per site).

Table 1
Age, sex, and breed characteristics of horses in OC-affected and normal control groups.

	OC-affected	Normal controls
Age	Median (range): 4.5 months (1–5)	Median (range): 4 months (3–6)
Sex	3M, 3F	1M, 7F
Breed	3 QH cross, 1 TB, 1 POA, 1 Lipizzaner	7 QH cross, 1 POA

2.4. RNA isolation

PicoPure RNA Isolation Kit (Arcturus Bioscience, Mountainview, CA, USA) was used for RNA isolation of LCM samples with slight modifications. Briefly, cell lysate from up to 8 caps was loaded onto a preconditioned RNA-purification column. The column was then washed and treated with RNase-free DNase prior to RNA elution from the column. RNA quality control was performed using an Agilent 2100 Bioanalyzer and the RNA 6000 Pico LabChip kit (Agilent Technologies, Santa Clara, CA, USA).

2.5. Real-time quantitative polymerase chain reaction

Following RNA isolation from laser-captured cells, reverse transcription was performed to create cDNA, using random hexamers. In order to maximize the amount of cDNA available from these limited samples, logarithmic pre-amplification with equine specific primers and the Taqman PreAmp Master Mix kit (Applied Biosystems, Foster City, CA) was performed. Equine specific primers and probes for the TaqMan® system were previously designed (Collagen type I, Collagen type IIB, Collagen type X, Osteocalcin, SOX 5, SOX 9, MMP-1, MMP-3, COMP, Jagged2, Ephrin-β2, Gremlin1, PRG4, Runx2, Aggrecan, Aggrecanase), meeting specific criteria of Primer Express software version 2.0b8a (Applied Biosystems, Foster City, CA). Real-time quantitative PCR of diluted pre-amplified cDNA samples (1:20) was then performed according to standard protocol, using the ABI StepOnePlus real-time PCR system and software (Applied Biosystems, Foster City, CA) (Kinsley et al., 2015). Probes were labeled with a reporter dye, FAM (6-carboxyfluroscein), and a quencher dye, TAMRA (6-carboxy-teramethylrhodamine). For each experimental sample the amount of target cDNA was determined by a relative standard curve, using the same calibrator (cDNA sample having highest gene expression across multiple genes) for all experiments. PCR was performed in duplicate using 20 μl final reaction mixture of 2× Taqman® Gene Expression Master Mix, 250 nM probe, 900 nM forward and reverse primers, and 7.5 μl pre-amplified sample cDNA. 18S RNA was used as the housekeeping gene for normalizing gene expression. After a two-minute incubation at 50 °C activating uracil-DNA glycosylase (UDG) and ten-minute incubation at 95 °C to deactivate UDG and activate AmpliTaq®Gold DNA polymerase, 40 PCR cycles of 15 s of 95 °C followed by 1 min of 60 °C were run.

2.6. Immunohistochemistry

Immunohistochemistry (IHC) was performed on paraffin-embedded osteochondral sections using mouse monoclonal primary antibodies for Osteocalcin (cat#MAB1419, R&D Systems, Inc., Minneapolis, MN) and Collagen type IIB (clone M2139, Thermo Fisher Scientific, Waltham, MA) and rabbit polyclonal primary antibodies for Lubricin (ab28484, Abcam, Cambridge, UK) and SOX9 (PA5-23383, Thermo Fisher Scientific, Waltham, MA). The Supersensitive Link-label Multilink Immunohistochemistry System (Biogenex, San Ramon, CA) was used for this procedure. Negative procedural controls were confirmed by substituting non-immune IgG serum in place of primary antibody. Following deparaffinization, osteochondral sections (6 OC, 8 normal) were

incubated at 37 °C for 60 min under a solution of testicular hyaluronidase to expose the antigen. Endogenous peroxidases were quenched with peroxide block. Non-immune goat serum was applied for 30 min (polyclonal primary antibodies only), and the primary antibody was applied for 60–90 min at room temperature. Secondary biotinylated multilink antibodies (Biogenex, San Ramon, CA) were applied, followed by labeling with streptavidin conjugated peroxidase, and then applying diaminobenzidine tetrachloride (DAB) as a chromogen for production of color product. The sections were counterstained with Harris hematoxylin and mounted for microscopy.

2.7. Immunohistochemistry scoring

Using the previously described HSCORE scoring system (Brun et al., 2012; Schatz et al., 2012) samples were evaluated by two blinded investigators (SAS, SKG). Scoring of each sample was determined for specific cell populations (chondrocytes surrounding the cartilage canals and chondrocytes adjacent to the osteochondral junction) by using the equation HSCORE = Σi i * Pi which calculates the sum of the percentage of positive staining cells or matrix (Pi) at each intensity multiplied by its respective intensity score (i). Staining intensity scores were from 0 to 3 as follows: 0 = no staining, 1 = mild staining, 2 = moderate staining, and 3 = strong staining. Scores were averaged at each location by both investigators. Superficial, middle, and deep cartilage layers were also scored, averaged, and the HSCORE calculated for each cartilage layer. In addition, the total percentage of immunopositive cells and matrix was determined by adding the averaged percentages of staining intensities 1, 2 and 3 for each layer and cell population.

2.8. Statistical analysis

Quantitative comparisons from real-time PCR assays were compared for each site (cartilage canal, osteochondral junction) between OC and normal horses using a Wilcoxon rank sum test ($P \leq 0.05$).

Immunohistochemistry scores were compared between OC and normal horses using a Wilcoxon rank sum test ($P \leq 0.05$). Samples were placed into two groups for statistical analysis; normal or osteochondrosis-affected. Of those samples that had a paired contralateral limb, samples were averaged for a single total value to be assigned to each individual foal (all contralateral limb pairs were either both OC or both normal). Values from the averaged HSCOREs and the averaged total percentage of positive cells/matrix for the superficial, middle, and deep layers, osteochondral junction, cartilage canals, and bone were evaluated. Extracellular matrix and cellular expression were evaluated separately for each layer.

3. Results

3.1. Real-time quantitative polymerase chain reaction

There was significantly decreased gene expression of Collagen type IIB ($P = 0.01$), Aggrecan ($P = 0.03$), and SOX-9 ($P = 0.04$) in chondrocytes surrounding the cartilage canals of early osteochondrosis-affected samples compared to normal controls (Fig. 1). Similarly, in chondrocytes along the osteochondral junction, there was significantly lower expression of Collagen type IIB ($P = 0.04$) and Lubricin ($P = 0.01$) in osteochondrosis samples compared to normal controls (Fig. 2). There was no difference in gene expression of Collagen type I, Collagen type X, Osteocalcin, SOX-5, MMP-1, MMP-3, COMP, Jagged2, Ephrin-β2, Gremlin1, PRG4, Runx2, or Aggrecanase between OC affected and normal samples.

3.2. Immunohistochemistry

Protein expression for Sox-9 was observed throughout the extracellular matrix and cells of each cartilage layer (superficial, middle, and deep), surrounding cartilage canals, along the osteochondral junction, and bone (Fig. 3). Immunostaining for Sox-9 was mild within chondrocytes of each of the cartilage layers. Mild expression was noted sporadically in chondrocytes along the osteochondral junction. Protein expression for Sox-9 was found within osteoblasts and chondroclasts of newly formed bone. Mild immunostaining was observed in chondrocytes surrounding the cartilage canals. No statistically significant differences were seen in HSCORE values for Sox-9 in OC affected cartilage samples versus normal controls for either extracellular matrix or cells within the cartilage layers, surrounding the cartilage canals, along the osteochondral junction, and within bone (Fig. 3, Table 2). However, the total percentage of Sox-9 positive cells was higher in bone of OC affected samples (23 %) than normal controls (2 %) ($P = 0.04$).

Protein expression for PRG4 (lubricin) was observed throughout the extracellular matrix and cells of each cartilage layers (superficial, middle, and deep), surrounding cartilage canals, along the osteochondral

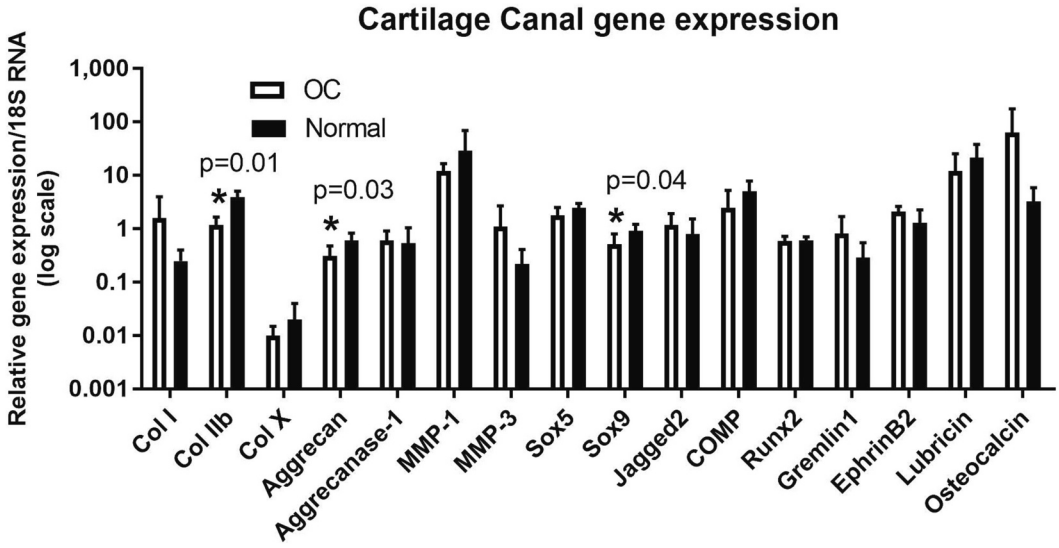

Fig. 1. Gene expression of laser-captured cartilage canal chondrocytes from 4 OC-affected and 5 normal foals using real-time PCR. Relative gene expression (log scale) was determined using a relative standard curve and dividing the target gene by 18S RNA. There was significant ($P \leq 0.05$) downregulation of Collagen type IIB, Aggrecan, and SOX-9 in OC samples compared to normal controls.

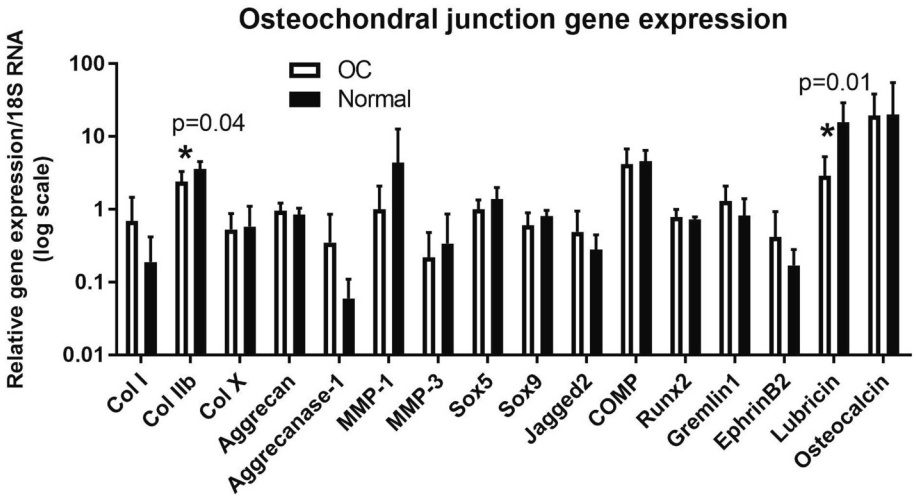

Fig. 2. Gene expression of laser-captured osteochondral junction chondrocytes from 4 OC-affected and 5 normal foals using real-time PCR. Relative gene expression (log scale) was determined using a relative standard curve and dividing the target gene by 18S RNA. There was significant ($P \leq 0.05$) downregulation of collagen type IIB and lubricin in early OC compared to normal controls.

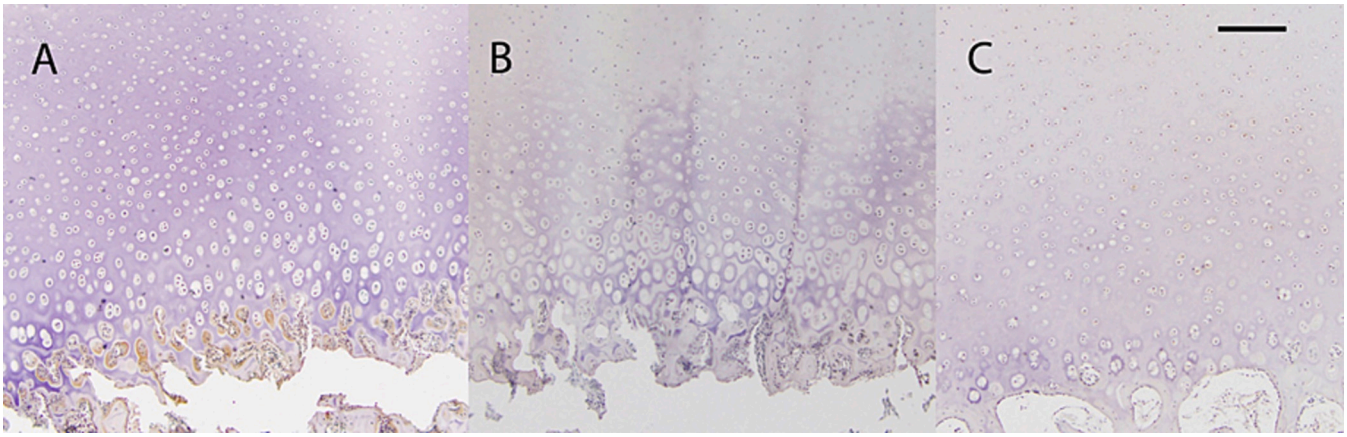

Fig. 3. Photomicrographs depicting protein expression for Sox-9 following immunohistochemical localization. A) Osteochondral sample of foal with confirmed OC showing moderate Sox-9 expression in chondrocytes along the osteochondral junction. B) Negative control for (A) following substitution of mouse monoclonal Sox-9 antibody with nonimmune serum. C) Osteochondral samples of normal foal (bar = 100 μm).

Table 2
Mean Sox-9 protein expression HSCORE ± SEM and mean total percentage of immunopositive matrix and cells.

Cartilage layer		OC (n = 6) HSCORE	Normal (n = 8) HSCORE	*P*-value	OC Total % positive	Norm Total % positive	*P*-value
Superficial	Matrix	0.71 ± 0.03	0.28 ± 0.02	0.11	43	18	0.07
	Cellular	0.20 ± 0.13	0.21 ± 0.16	0.89	12	14	0.35
Middle	Matrix	0.04 ± 0.04	0.01 ± 0.01	1.00	36	1	0.55
	Cellular	0.15 ± 0.13	0.08 ± 0.05	0.92	7	11	0.28
Deep	Matrix	0.00 ± 0.00	0.00 ± 0.00	1.00	0	0	0.50
	Cellular	0.19 ± 0.13	0.06 ± 0.02	0.54	10	6	0.44
Cartilage canals	Matrix	0.00 ± 0.0	0.00 ± 0.00	1.00	2	0	0.50
	Cellular	0.07 ± 0.04	0.01 ± 0.01	0.17	14	1	0.16
Osteochondral junction	Matrix	0.00 ± 0.00	0.00 ± 0.00	1.00	4	0	0.46
	Cellular	0.17 ± 0.13	0.03 ± 0.01	0.26	11	3	0.12
Bone	Matrix	0.12 ± 0.12	0.00 ± 0.00	0.43	9	0	0.13
	Cellular	0.40 ± 0.21	0.03 ± 0.01	0.08	23	2	0.04

junction, and bone (Fig. 4). Immunostaining within the extracellular matrix was observed for lubricin sporadically within the superficial and middle cartilage layers. Lubricin protein expression was found mainly within the deep cartilage layer, along the matrix interface of cartilage and bone in lacunae formed by chondroclasts. Mild to moderate expression was observed within the extracellular matrix surrounding cartilage canals. No extracellular matrix expression was observed within bone. Within cells, protein expression was observed throughout the cartilage layers, mainly expressed within cells of the deeper cartilage layer. Lubricin expression was also observed along the osteochondral junction, with no expression observed within bone cells. No statistically significant differences were seen in HSCORE values for lubricin in OC

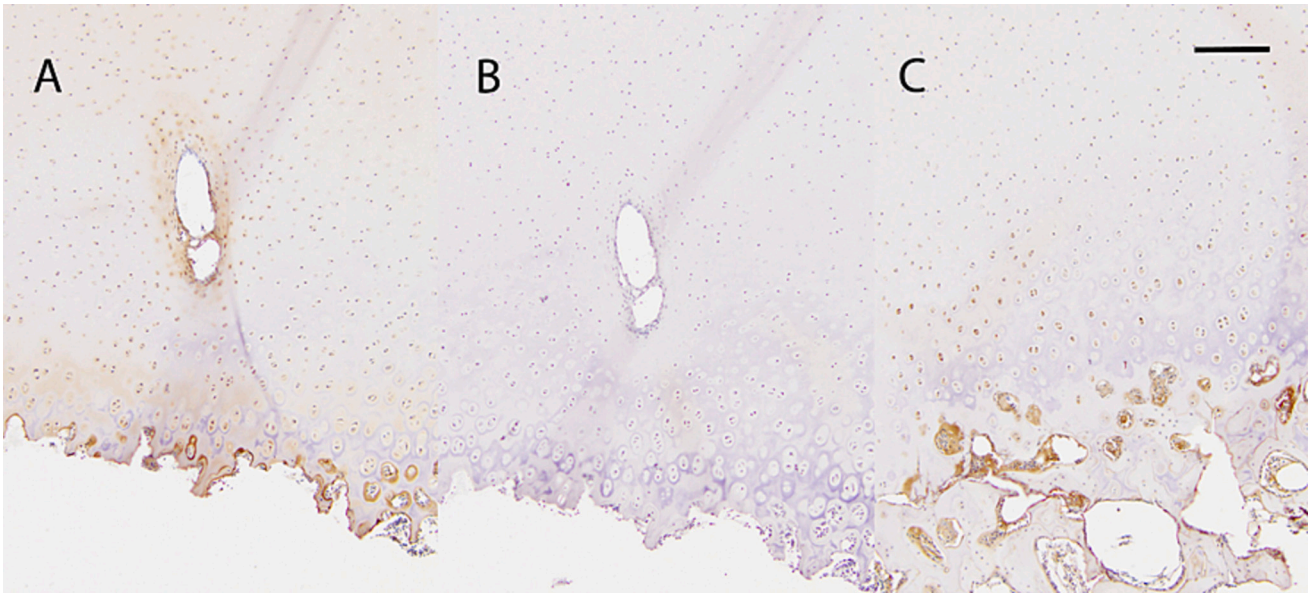

Fig. 4. Photomicrographs depicting protein expression for lubricin following immunohistochemical localization. A) Osteochondral sample of foal with confirmed OC showing moderate to strong lubricin expression in chondrocytes along the osteochondral junction and surrounding cartilage canals with mild to moderate expression throughout the extracellular matrix of the deeper cartilage layers. B) Negative control for (A) following substitution of rabbit polyclonal lubricin antibody with nonimmune serum. C) Osteochondral samples of normal foal (bar = 100 μm).

affected cartilage samples versus normal controls for either extracellular matrix or cells within the cartilage layers, surrounding the cartilage canals, along the osteochondral junction, and within bone (Fig. 4, Table 3). However, the total percentage of lubricin positive cells was lower in the superficial cartilage layer of OC affected samples (12 %) than normal controls (32 %) ($P = 0.04$).

Protein expression for osteocalcin was observed throughout the extracellular matrix and cells of each cartilage layer (superficial, middle, and deep), surrounding cartilage canals, along the osteochondral junction, and bone (Fig. 5). Sporadic osteocalcin expression was observed as mild to moderate immunostaining within chondrocytes throughout all layers of articular cartilage. Protein expression of osteocalcin was confined mainly to osteoblasts and chondroclasts in newly formed bone, as well as in chondrocytes of the deep cartilage layer and along the osteochondral junction. No notable extracellular matrix staining was noted. No significant differences were seen in HSCORE values for osteocalcin between OC affected cartilage samples and normal controls for either extracellular matrix or cells within the cartilage layers, surrounding the cartilage canals, along the osteochondral junction, and within bone (Fig. 5, Table 4). Similarly, no significant differences were found in total percentage of osteocalcin positive matrix and cells between OC affected and normal samples.

Protein expression for collagen type IIB was observed throughout the extracellular matrix and cells of each cartilage layer (superficial, middle, and deep), surrounding cartilage canals, and along the osteochondral junction (Fig. 6). No matrix or cellular expression was noted in bone. Collagen type IIB immunostaining was mild to moderate throughout most of the cartilage matrix, and cellular expression of collagen type IIB was mild throughout. No statistically significant differences were seen in HSCORE values for collagen type IIB between OC affected cartilage samples and normal controls for either extracellular matrix or cells within the cartilage layers, surrounding the cartilage canals, along the osteochondral junction, or within bone (Fig. 6, Table 5). However, the total percentage of collagen type IIB positive matrix expression was lower in the middle ($P = 0.005$) and deep ($P = 0.02$) cartilage layers of OC affected samples than normal controls.

4. Discussion

We expected to find downregulation of extracellular matrix gene and protein expression and upregulation of matrix degradation molecules within the osteochondral junction and in cells surrounding the cartilage canals of early osteochondrosis-affected samples. In partial support of our hypothesis, we found significantly decreased gene expression of collagen type IIB, aggrecan, and SOX-9 in chondrocytes surrounding the cartilage canals and decreased gene expression of PRG4 (Lubricin) and

Table 3
Mean lubricin protein expression HSCORE ± SEM and mean total percentage of immunopositive matrix and cells.

Cartilage layer		OC (n = 6) HSCORE	Normal (n = 8) HSCORE	*P*-value	OC Total % positive	Norm Total % positive	*P*-value
Superficial	Matrix	0.44 ± 0.10	0.79 ± 0.15	0.13	44	68	0.06
	Cellular	0.15 ± 0.07	0.39 ± 0.14	0.14	12	32	0.04
Middle	Matrix	0.02 ± 0.02	0.03 ± 0.03	1.00	2	3	0.69
	Cellular	0.10 ± 0.05	0.23 ± 0.08	0.32	8	17	0.21
Deep	Matrix	0.14 ± 0.09	0.13 ± 0.13	0.54	14	12	0.38
	Cellular	0.27 ± 0.12	0.56 ± 0.19	0.39	23	37	0.19
Cartilage canals	Matrix	0.24 ± 0.12	0.32 ± 0.15	0.52	24	32	0.26
	Cellular	0.43 ± 0.19	0.10 ± 0.08	0.41	45	10	0.21
Osteochondral junction	Matrix	0.18 ± 0.11	0.06 ± 0.06	0.32	16	2	0.16
	Cellular	0.47 ± 0.16	0.59 ± 0.19	0.64	36	43	0.34
Bone	Matrix	0.44 ± 0.10	0.79 ± 0.15	0.13	40	28	0.13
	Cellular	0.15 ± 0.07	0.39 ± 0.14	0.14	3	16	0.14

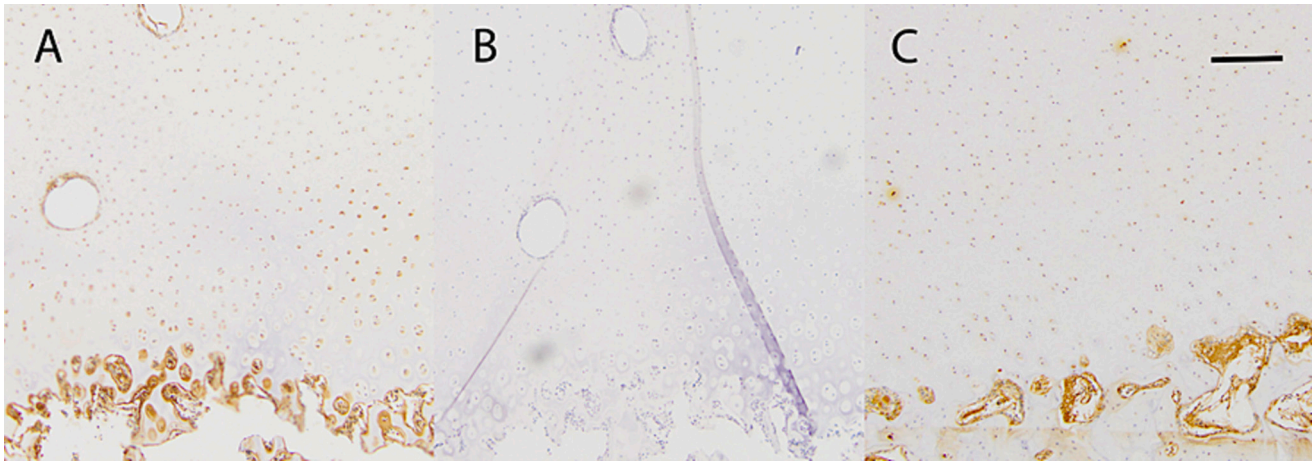

Fig. 5. Photomicrographs depicting protein expression for osteocalcin following immunohistochemical localization. A) Osteochondral sample of foal with confirmed OC showing moderate to strong osteocalcin expression in chondrocytes along the osteochondral junction and in within the deeper cartilage layers. B) Negative control for (A) following substitution of mouse monoclonal osteocalcin antibody with nonimmune serum. C) Osteochondral samples of normal foal showing similar results (bar = 100 μm).

Table 4
Mean osteocalcin protein expression HSCORE ± SEM and mean total percentage of immunopositive matrix and cells.

Cartilage layer		OC (n = 6) HSCORE	Normal (n = 8) HSCORE	*P*-value	OC Total % positive	Norm Total % positive	*P*-value
Superficial	Matrix	1.23 ± 0.35	0.69 ± 0.25	0.26	70	44	0.06
	Cellular	0.46 ± 0.12	0.58 ± 0.25	0.59	25	28	0.46
Middle	Matrix	0.17 ± 0.17	0.00 ± 0.00	0.42	14	0	0.47
	Cellular	0.42 ± 0.07	0.52 ± 0.08	0.21	38	37	0.42
Deep	Matrix	0.00+/− 0.00	0.00 ± 0.00	1.00	0	0	0.50
	Cellular	0.92 ± 0.13	1.03 ± 0.19	0.88	65	65	0.42
Cartilage canals	Matrix	0.0 ± 0.00	0.00+/− 0.00	1.00	0	0	0.50
	Cellular	0.64 ± 0.23	0.41 ± 0.19	0.60	41	30	0.26
Osteochondral junction	Matrix	0.00 ± 0.00	0.00+/− 0.00	1.00	0	0	0.50
	Cellular	0.90 ± 0.15	0.99 ± 0.16	0.68	68	68	0.49
Bone	Matrix	0.12 ± 0.12	0.00 ± 0.00	0.43	6	0	0.38
	Cellular	1.63 ± 0.42	1.47 ± 0.17	0.72	66	68	0.46

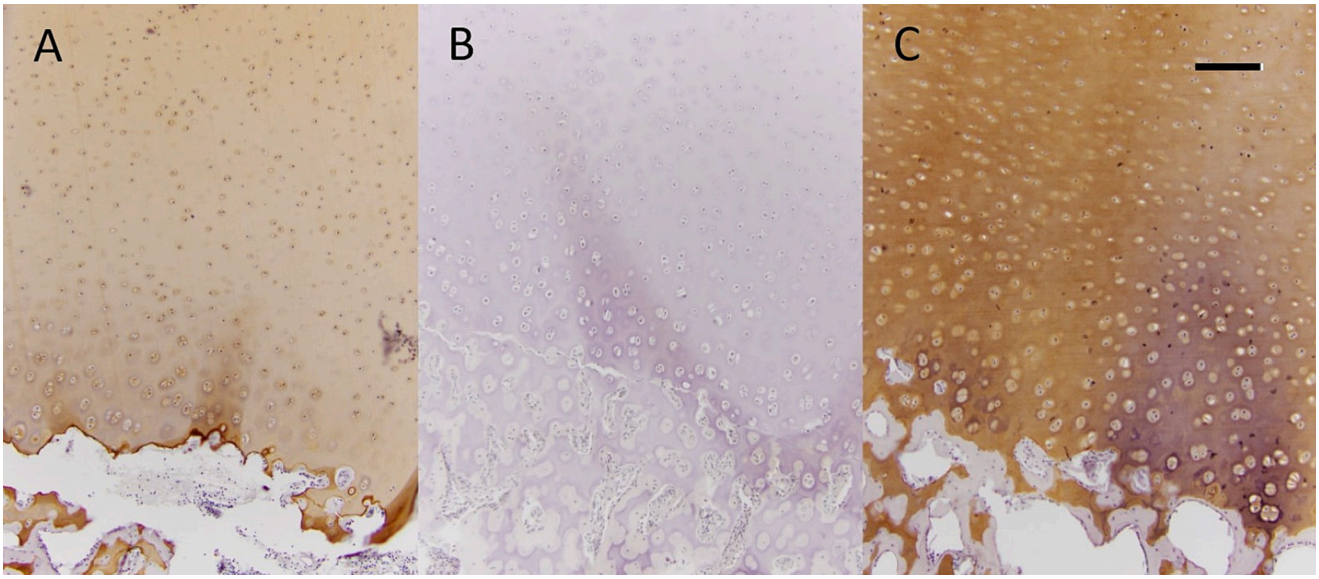

Fig. 6. Photomicrographs depicting protein expression for collagen type IIB following immunohistochemical localization. A) Osteochondral sample of foal with confirmed OC showing moderate collagen type IIB expression throughout the extracellular matrix of the articular zone with strong expression localized to the hypertrophic zone B) Negative control following substitution of mouse monoclonal collagen type IIB antibody with nonimmune serum. C) Osteochondral samples of normal foal showing moderate to strong collagen type IIB expression in the cartilage matrix (bar = 100 μm).

Table 5
Mean collagen type IIB protein expression HSCORE ± SEM and mean total percentage of immunopositive matrix and cells.

Cartilage layer		OC (n = 6) HSCORE	Normal (n = 8) HSCORE	*P*-value	OC Total % positive	Norm Total % positive	*P*-value
Superficial	Matrix	1.21 ± 0.29	0.84 ± 0.15	0.24	63	65	0.39
	Cellular	0.33 ± 0.14	0.26 ± 0.14	0.69	34	42	0.29
Middle	Matrix	0.87 ± 0.19	0.69 ± 0.15	0.55	47	86	0.005
	Cellular	0.83 ± 0.16	0.83 ± 0.18	0.80	16	16	0.45
Deep	Matrix	0.0 ± 0.21	0.82 ± 0.12	0.28	57	83	0.02
	Cellular	0.48 ± 0.10	0.47 ± 0.11	0.84	61	68	0.33
Cartilage canals	Matrix	0.57 ± 0.08	0.32 ± 0.12	0.17	77	73	0.07
	Cellular	0.04 ± 0.02	0.16 ± 0.11	0.70	29	27	0.40
Osteochondral junction	Matrix	0.55 ± 0.31	1.27 ± 0.10	0.15	67	100	0.30
	Cellular	0.15 ± 0.09	0.24 ± 0.10	0.64	76	68	0.18
Bone	Matrix	0.00 ± 0.00	0.00 ± 0.00	1.00	24	23	0.27
	Cellular	0.00 ± 0.00	0.00 ± 0.00	1.00	15	47	0.38

collagen type IIB in chondrocytes along the osteochondral junction in OC-affected samples. In addition, we found significantly lower collagen type IIB total matrix percentages in the middle and deep cartilage layers and lower lubricin total cellular percentage in the superficial layer of OC samples. However, contrary to our hypothesis, we found higher Sox-9 total cellular percentage in bone of OC samples and no significant differences in matrix degradation molecules or HSCORE protein expression at any locations between normal and OC-affected samples in our study,

Collagen type IIB cleavage has previously been shown to be altered in the extracellular matrix of early equine osteochondrosis (Laverty et al., 2002). In addition, a reduction in total type II collagen content and number of collagen crosslinks is found in early osteochondrosis affected foals (van de Lest et al., 2004). In our study, we discovered a decrease in gene expression of collagen type II in cells along the osteochondral junction and near cartilage canals in OC-affected foals. Significantly lower matrix percentages of type II collagen in the middle and deep cartilage layers of OC samples further support these findings, although no differences were found in HSCORE values. Interestingly, the total percentage of collagen type IIB positive matrix trended higher in OC samples near cartilage canals, but did not reach significance ($P = 0.07$).

Our findings differ from previous studies in which an increase in collagenase accompanies an increase in collagen degradation in osteochondrosis of horses (Laverty et al., 2002) and pigs (Wardale and Duance, 1994). In contrast, we found no upregulation of matrix degradation enzyme expression in our laser capture microdissection samples. A previous study in 1 to 6 month old horses found an upregulation of MMP-3 and MMP-13 gene expression in OC-affected full thickness cartilage samples and increased MMP-13 gene expression surrounding cartilage canals (Riddick et al., 2012). In the current study, we did not find increased MMP-1 or MMP-3 gene expression in chondrocytes surrounding cartilage canals or along the osteochondral junction in OC-affected foals. The differing results for MMP-3 expression between the two studies is likely due to differences in sample types and locations (full thickness cartilage vs. laser captured cells).

In our study, reduced aggrecan gene expression was found in chondrocytes surrounding the cartilage canals of OC-affected foals. Aggrecan, a proteoglycan bound to hyaluronic acid, is a critical component of cartilage structure and the function of joints (Statham et al., 2021). Due to its expression by chondrocytes, aggrecan is often used as an indicator of chondrocyte activity and may play an important role in early mineralization (Hoogen et al., 1999). Early studies have shown that the size and shape of the proteoglycan may become altered in order to allow for new bone formation to occur, but that there is no net loss of aggrecan in normal mineralization (Pool, 1993). Our findings of reduced aggrecan gene expression in chondrocytes surrounding cartilage canals in OC-affected foals may predicate failure of the cartilage canals and ischemic necrosis (Laverty and Girard, 2013; Olstad et al., 2013).

A concurrent decrease in SOX-9 gene expression in chondrocytes surrounding the cartilage canals of OC-affected foals may support altered chondrocyte differentiation in this region. SOX-9 inhibits chondrocytes entering into hypertrophy, balancing stimulatory effects of SOX-5 and SOX-6 on maturation of chondrocytes (Ikeda et al., 2005). SOX-9 has also been shown to directly activate genes for aggrecan and collagen type IIa, in conjunction with SOX-5 and SOX-6, yielding downstream effects on cartilage formation (Lefebvre et al., 2001). Reduced SOX-9 gene expression surrounding cartilage canals of OC-affected cartilage may contribute to altered cell signaling and differentiation in this region. In addition, a higher total percentage of Sox-9 positive cells in the bone of OC samples may indicate dysregulation of Sox-9 protein expression in other areas.

Interestingly, reduced PRG4 (lubricin) gene expression was found in cells along the osteochondral junction. PRG4 encodes the mucinous glycoprotein lubricin and functions as a boundary lubricant in synovial fluid (Waller et al., 2017). Lubricin has been shown to delay the progression of osteoarthritis in rodent models (Teeple et al., 2011) and reduce cartilage degeneration following meniscal injury in minipig models (Waller et al., 2017). In naturally occurring osteoarthritis in the horse, PRG4 gene expression is increased in the synovial membrane but decreased in cartilage (Reesink et al., 2017). Lower total percentage of lubricin positive cells in the superficial cartilage layer of OC samples further supports its potential role in OC.

Although no difference in BGLAP (osteocalcin) gene expression was apparent in OC samples of our study, immunohistochemistry was performed for osteocalcin. A previous study reveal the presence of OC lesions to be significantly correlated to elevated serum osteocalcin concentrations in foals (Donabedian et al., 2008). Increased bone metabolism due to the presence of osteochondrosis in foals as early as 2 weeks old can be detected before radiographic changes occur at 5.5 to 11 months of age (Donabedian et al., 2008). In our study, protein expression of osteocalcin is confined mainly to osteoblasts and chondroclasts in newly formed bone, as well as in chondrocytes of the deep cartilage layer and along the osteochondral junction. No observable or statistically significant differences were noted between OC affected and normal control osteochondral samples.

Variations can be seen between gene and protein expression as described in other studies (Riddick et al., 2012). Our study was conducted using samples collected from foals mainly around 4 months of age. This critical age for growth has been shown to be significant in the development of histological lesions compatible with osteochondrosis in a multitude of studies (Carlson et al., 1995; Olstad et al., 2008; Olstad et al., 2011). However, taking into consideration the highly dynamic nature of these molecular processes it is impossible to eliminate the temporal variances at the time samples were collected. By evaluating specific sites within the trochlear ridges of the femoral condyle, and with the aid of laser capture microdissection, it was possible to evaluate very specific areas within the cartilage including cells surrounding the cartilage canals and along the osteochondral junction in order to eliminate spatial discrepancies.

Limitations of the study included a targeted rather than comprehensive evaluation of genes expressed in the extracellular matrix,

mainly due to limited amount of cDNA from laser-captured samples. In addition, only two cell populations were collected via laser-capture microdissection (~400–800 cells per cell population) due to having limited time to complete the laser capture procedure without sample degradation. We did not evaluate protein expression for every gene due to financial constraints. It is important to note that gene and protein expression studies do not address longitudinal changes or activity of molecules within the region. The small number of samples likely contributed to type I error and may have limited the ability to determine differences between OC and normal samples.

5. Conclusions

In conclusion, we found expected downregulation of several key extracellular matrix constituents in cells surrounding the cartilage canals, along the osteochondral junction and cartilage layers in OC samples. However, no change in matrix metalloproteinase gene expression was found between OC and normal samples. It is possible with a greater number of samples that more differences might become apparent between OC and normal cartilage. Thus, further studies are warranted to confirm or refute these findings.

CRediT authorship contribution statement

S.K. Grissom: Writing – original draft, Investigation, Visualization, Funding acquisition. **S.A. Semevolos:** Conceptualization, Methodology, Validation, Formal analysis, Resources, Writing – review & editing, Supervision, Funding acquisition, Investigation, Visualization. **K. Duesterdieck-Zellmer:** Investigation, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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